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Searching for Possible Association between Six Microsatellite Markers and Suppression of Mite Reproduction (SMR) Trait in Honey Bees

Research Article

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ABSTRACT

There are several candidates' heritable traits in honey bees that can be selected to make colonies genetically resistant to Varroa mites. This study was conducted to find an association between six microsatellite markers and suppression of the mite reproduction in honey bees. The study included two-phases. In the first phase, phenotypic measurement was done based on Varroa mite reproduction success and included direct counting of the number of cells containing the mother mite that have successfully reproduced (fertility), the total number of offspring, and the number of offspring mites per mother mite (fecundity). In the second molecular genotyping phase, the polymerase chain reaction (PCR) was amplified using specific primers to investigate the polymorphism of six primers. The results showed that HQ7622 and KO430 loci expressed the highest (6 alleles) and lowest (1 allele) number of alleles, respectively. Interestingly, the HQ7622 microsatellite marker was significantly associated with all studied traits ($P<0.05$). The effect of the HQ7691 locus on the number of mother mites and the rate of mite infestation was significant ($P<0.05$). Furthermore, UN086 didn't affect any of the measured phenotypic characteristics. The UN334d locus significantly affected the number of mother mites and the rate of mite infestation ($P<0.05$). Effect of UN391 locus on maternal mite count ($P<0.05$), offspring mite count ($P<0.01$), total mite count ($P<0.01$), fertility ($P<0.05$), and the rate of mite infestation ($P<0.05$) was significant. On this basis, we demonstrated the importance of certain microsatellite markers for the genetic identification of bee colony resistance to Varroa mites.

KEY WORDS honey bee, microsatellite markers, suppression of mite reproduction, Varroa mite.

INTRODUCTION

The topic of Varroa mites in beekeeping has received a lot of critical attention so far. Understanding the complexity of the interaction of the host genome with the Varroa mite is crucial, since contamination of the hive with the mite decreases the biological vitality of the bees. Recently, considerable literature has emerged around the topic of spread of Varroa mites and its negative consequences. Due to the adverse effects of the large-scale application of synthetic chemicals against mites, attempts are being made to use mite-resistant bee colonies (Fries *et al.* 1994). The literature review shows that there are several candidate heritable traits in bees that can be selected to obtain colonies that are genetically resistant to Varroa mites (Kirrane *et al.* 2011). One of these traits, recently introduced and directly related to mite resistance, is called mite reproductive suppression (SMR). Research into SMR inbreeding has a long history because, to sum up this terminology, bees disrupt or inhibit the reproduction of mites in larval cells.

This reduces the growth of the mite population. SMR occurs through two mechanisms: First, worker bees cause pupal cells to open and close these caps for a limited time. Some authors have also pointed out that opening an infected cell for a certain period of time, exactly at the time of bee larval development, has a negative effect on Varroa mite reproductive success (Kirrane *et al.* 2011).

The authors bring some information about the background of the problem and another interesting mechanism is the inhibition of mite reproduction through the secretion of some volatile compounds by the growing larvae that inhibit the reproduction of Varroa mite. This mechanism is a direct route to mite resistance, in which its infected larvae inhibit mite proliferation in the cell (Fries *et al.* 1994). Mite reproduction is closely coordinated with pupal development, and various components of the larval cuticle are responsible for the initiation of mite oviposition (Rosenkranz and Garrido, 2004; Garrido *et al.* 2003).

A number of authors have recognized that in European bee subspecies, typically 20.5% of Varroa mites become sterile after invading worker larval cells (Rosenkranz *et al.* 2010). Over time, an extensive body of literature has developed on high levels of reproductive dysfunction as an inherited trait of worker bees, referred to as mite reproductive suppression (Harbo and Harris, 1999). For example, research has shown that in some European populations of *Apis mellifera* naturally selected for resistance to Varroa mites, the proportion of mites that do not breed is significantly increased (50-40%) (Locke and Fries, 2011). Mondet *et al.* (2020) screened 414 colonies across the entire European continent and observed a great variability of SMR across the different honey bee populations, with a mean proportion of non-reproducing mites reaching an overall score of 32.8%, and close to 16% of colonies exceeding a score of 50%.

Solignac *et al.* (2004) prepared a consistency map using microsatellite markers. The researchers mapped a total of 541 locations, of which 474 were microsatellite locations. Twenty-four groups estimated continuity, of which 5 were subgroups (7.1-22.8 cM) and 19 were main groups (76.5 cM).

The most comprehensive study on SMR, was conducted by Behrens *et al.* (2011) using backcross population. In summary, research focused on 488 microsatellite markers located on all 16 bee chromosomes numbers (Bulk Segregation Analysis) and two resistant and susceptible male pupae were genotyped. Based on the BSA, followed by the determination of individual genotype, three interesting regions located on chromosomes 4, 7 and 9 were identified, indicating the attachment of at least one marker to the trait of resistance. They also identified two genes, including the first orthologous of the foxo gene (GB11764), the transcript of the insulin signaling pathway, on chromosome 7, and the second orthologous of the Drosophila gene, futsch (GB11509). Gebremedhn *et al.* (2023) investigated the expression profiles of some key molecular markers involved in resilience to varroa infestation and found significantly higher gene expression of the odorant binding protein, OBP14, in the antennae of Ethiopian bees compared to Belgian bees.

In Iran, various traits of mite resistance have been studied by many researchers. Including hygienic behavior, grooming behavior, duration of post capping period, etc., and the results of those research indicate the existence of mite resistance traits in some bee colonies in the country. Sepehri *et al.* (2023) surveyed single nucleotide polymorphism (SNP) markers of NorpA2 candidate gene and showed the existence of three specific differences in sequence in the form of SNP (C/T) at position 308 and the form of SNP/deletion at positions 504 and 563 of the nucleotide sequence region in the promoter between the SUS and RES groups. However, the trait of SMR in Iran was studied for the first time in present report. One of the features of present study was the simultaneous study of phenotypic and genotypic and the study of the relationship between them and the use of male pupae for phenotypic and genotypic recordings. With this motivation, the main goal of present report was to search for possible associations between six microsatellite markers and suppression of mite reproduction traits in honey bees.

MATERIALS AND METHODS

Bees and sampling process

To perform this research, in 2013, 50 Iranian honey bee colonies (*Apis mellifera meda*) were choose from commercial apiaries in East Azarbaijan and, in order to obtain a mite resistant population, were maintained for three consecutive years without fighting Varroa mites. These colonies were housed in standard open- bottomed Langstroth hives.

The open-bottomed hive allows the mites that have fallen on the hive floor to not be able to return to the adult bees or their pupae. In the first year, 5, in the second year 31 and in the third year 25 colonies were lost. Every spring, colonies that survived the previous winter without fighting mites were used to form the next generation. In this way, by rearing the queen and the drone from the remaining colonies and controlled mating, the remaining queen colonies were replaced and a new colony was established instead of the lost colonies. At the end of the third year, the mite infestation rate of these colonies was relatively low, ranging from 7% to 13% (percentage of adult bee infestation). In this method, a survivor line was created in terms of mite infestation.

A pure Carniolan (*Apis mellifera carnica*) queen (ID: B125) was purchased from Alvand Queen Breeding Company in 2015 and properly introduced into an established foster colony. To obtain hybrid colonies, several virgin queens were raised from one-day-old larvae from Carnica line. These queens naturally crossed with the males of our survivor colonies. New colonies were formed and mated queens were introduced to these colonies. The following year, a number of these colonies were selected as mothers and re-crossed with the males of our survivor colonies (backcross), and mating queens were obtained (Figure 1). These queens were introduced and established in new colonies and drone pupae of three backcrossed colonies were phenotypically and genetically evaluated for SMR. Figure 1 indicated an experimental design to produce drones for the measurement of SMR record.

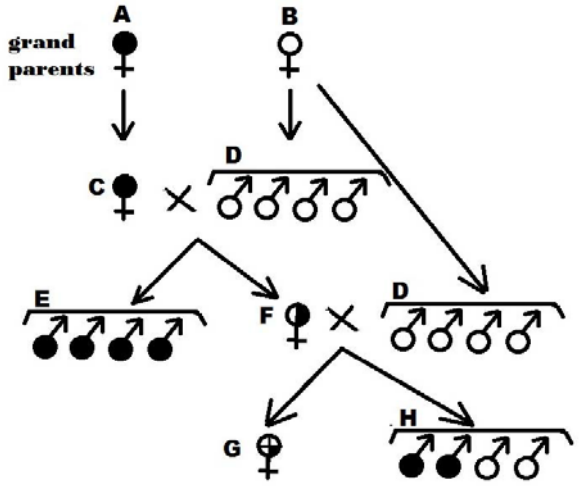

Figure 1 Crossing design to produce drones for measurement of SMR record. A: pure grandmother colony (Carniolan); B: drone producing grandfather colony (survivor); C: virgin daughter queen of A; D: drones produced by B; E: drones produced by Queen C that mated to D drones, due to haploidic they inherit only their mother genes; F: daughter of C Queen and D drones (F1); G: F2 virgin queen and H: drones produced by F1 colony or H1 drones

Phenotypic measurements

The reproductive success of Varroa mites is determined in three ways: 1) Count the number of cells containing the mother mite that have succeeded in reproducing (fertility). 2) A total number of offspring and 3) A number of offspring mites per mother mite (fecundity). Firstly, 1-2 drone combs were placed in the backcross colonies. 15 to 18 days later, each pupa cell was opened using a needle. The contents of each cell were emptied on a greaseproof white paper and the number of mother and offspring mites in the cell and on the pupae were examined and counted using forceps and a magnifying glass (Lee *et al.* 2010). At this stage of bee male pupation, the mother mites are dark brown and the offspring mites are lighter (white to light brown) and therefore easily recognizable and countable. At least 100 male pupae from each hive and a total of 563 were, phenotypically, examined and recorded.

Calculation of infestation of male pupae was achieved by (Dietemann *et al.* 2013):

Infection percent= (number of infested cells/number of inspected cells) ×100

The success of mite reproduction is tested by determining the familial structure of the mite in the infected cell. Different aspects of mite reproduction can be measured. Inside each pupa cell, the following information can be collected: sex, stage of development, and viability. Dead and live mites can also be detected if the larval cells are freshly examined. To check for mite reproduction, the test larval cell must first be emptied and tested as follows: Using a pair of pliers, the pupa cell cap is carefully opened and the cell walls are pushed aside. The pupae are removed from the cell using forceps. It is best to take the pupae when removing it from the neck area. The pupae are placed on a white oiled cardboard. Then all parts of the pupa are examined using a loop and the number of mother and offspring mites is counted. Records of live mother mites and number of live offspring mites were recorded in the studied cells.

If the pupa cells contained at least one mother mite and one live offspring mite, it was considered a reproductive mite. This was obtained by dividing the number of reproductive mites by the number of cells that contained at least one mite and then multiplying it by 100% of reproducing mites (Dietemann *et al.* 2013). Another factor studied in this experiment, which was of great importance and was measured, was the intensity of mite reproduction. Mites with more than 3 offspring were also recorded separately. The percentage of mites that had more than 3 offspring was calculated by dividing the number of cells containing at least one mother mite and 3 offspring mites by the total number of infected cells and multiplying it by 100 (Behrens *et al.* 2011). Fecundity of *Varroa destructor* is one of the most important fitness parameters that affect mite population dynamics and infestation, especially in male larvae. Fecundity is the number of offspring per mite. In this experiment, the number of offspring in each cell was divided by the number of mother mites in it and was recorded as a Fecundity record

Molecular analysis

The pupae of the examined male cells were placed in individual sampling containers containing 70% ethanol alcohol and transferred to the Biotechnology Laboratory of the Research and Training Center for Agriculture and Natural Resources of East Azerbaijan in the presence of dry ice.

Samples were maintained at -20 ˚C until DNA extraction. Genomic DNA extraction was performed by the modified CTAB method. Agarose gel method was used to determine the quantity and quality of DNA. Polymerase chain reaction (PCR) was amplified using specific primers to investigate the existence of polymorphism. Table 1 illustrated characteristics and 6 investigated SSR primers.

PCR amplification was performed in a total volume of 15 µL containing master mix kit (Ampliqon, Denmark) 7.5 µL master mix 2X, 1 pmol of each primer (Forward and Reverse), and 4.9 µL ddH_2O, and 1.5 µL of genomic DNA (all these steps were done on ice). Model of PCR Machin for amplification of fragment was Biometra Company and the PCR Program was used in the PCR machine to replicate the STR loci of the Touch-down PCR specific program protocol was designed to simultaneously amplify the locus and minimize nonspecific and starter bands. The "Touchdown" PCR protocol was used with initial denaturation of 95 ˚C for 8 min, followed by the first stage of amplification of 12 cycles involving a denaturation step at 94 ˚C for 1 min, annealing based on reducing the temperature from 68 ˚C to 52 ˚C for 40 sec, and extension at 72 ˚C for 30 sec, and finally, the final reproduction temperature was set at 72 ˚C for 8 min. The PCR products were determined on 10% polyacrylamide gels, stained with silver nitrate 0.1% in 1X TAE buffer. Allele sizes were estimated using the 11 lines (25-755-bp) ladder (Life science Company).

Statistical analysis

The number of genotyped samples was 117 drone pupae. The phenotype of these samples was also measured. Data were analyzed using SAS (SAS, 2003). Due to the fact that after statistical transformations such as rooting, logarithm, etc., the distribution of the residuals of linear models did not become normal. It was decided to use the Chi-square test to analyze the data.

RESULTS AND DISCUSSION

Table 2 addressed descriptive statistics of measured traits in male pupae obtained from back crossed colonies.

As can be seen in Figure 2, the position of the microsatellite showed the polymorphism well in each of the samples. The size of the marker next to each gel makes it possible to identify the band size. In Figure 2, each column represents the genotype of a male pupa, and due to the n chromosomal nature of the males, a band is considered in each individual. Here, bands with reasonable distances from each other are considered different alleles.

Study of 6 loci showed that HQ7622 locus with 6 genotypes had the highest number of alleles and KO430 locus with one allele had the lowest number of alleles.

As shown in Table 3, the effect of HQ7622 locus on all measured traits is significant. The effect of HQ7691 locus on the number of mother mites ($P<0.05$) and the rate of mite infestation was significant ($P<0.05$). The effect of UN086 position on any of the traits is not significant. The effect of UN334d locus on the number of mother mites ($P<0.05$) and the rate of mite infestation was significant ($P<0.05$). Effect of UN391 locus on number of mother mites ($P<0.05$), number of offspring mites ($P<0.01$), the total number of mites ($P<0.01$), fecundity ($P<0.05$) and rate of mite infestation ($P<0.05$) is significant.

Table 3 displays P-value of different traits in relation to different loci, calculated from a Kruscal-Wallis Test. Analyzes performed to investigate the existence of any significant differences between phenotypic and genotypic data showed that all 5 traits measured showed a significant relationship with a number of loci. The results of PCR and examination of acrylamide gel photographs showed that the K0429 locus showed only one band and no variation was observed between the samples, so this locus was not used in statistical analysis. However, review of sources showed that this locus with a physical position of 4.272 Kb is located on chromosome 4 of bees and is one of the suggested loci for mite reproductive disorders (Behrens *et al.* 2011).

None of analyzes showed any association between UN086 status and any of the measured traits. This marker is located on chromosome 9 and the physical position of 2/167 Kb and has been reported in the studies of Behrens *et al.* (2011) as one of the candidate sites for mite reproductive disorder, but in the present study there is no relationship between this locus and the trait. This locus, along with the KO430, which showed no genetic diversity, is out of the question.

The remaining four loci in statistical analyzes showed a significant relationship with the measured traits. The HQ7691 locus with a physical position of 2.167 Kb is located on chromosome 7 and the results of data analysis showed that there was a significant relationship between this locus and some of the measured traits such as the number of mother mites in each cell are seen. This locus has been studied by Behrens *et al.* (2011), but in the report of these researchers, no significant relationship was found between this locus and the phenotype of mite reproductive disorder.

The UN334d locus with a physical position of 6/214 Kb is located on chromosome 7 and the results of data analysis showed that a significant relationship between this locus and some of the measured traits, including the number of mother mites in each cell and infection is seen. In QTL mapping studies conducted by Behrens *et al.* (2011), no significant relationship was found between this position and the trait of Varroa mite reproduction disorder.

Table 1 Primers, name, sequences, and length (Adopted from Behrens *et al.* 2011)

Name	Primer sequence	Length(bp)
UN086	5′-CCAATGAATGGGTAAATCTAGCTC-3′ F	24
	5′-TGTAGAATTCCATTGGCAACG-3′ R	21
UN334d	5′-TTACGATTGGGAACCGGG-3′ F	18
	5′-CCCAAACAATCGGAGGCA-3′ R	18
HQ7691	5′-CGGGAGCAACAACGAAGAAGGAACT-3′ F	25
	5′-CGCGGTGCCCCTTTTTACAGTAATC-3′ R	25
HQ7622	5′-TCGATGCGGCGAAAATCTCCTCTTT-3′ F	25
	5′-TGCATGGAGTTCGACTTGCAAAGGT-3′ R	25
K0429	5′-CTCTTGATTGAAAGAAACTCGCC-3′ F	23
	5′-AATAACGGTGTCATGCCGC-3′ R	19
UN391	5′-GTCGAATGGCTAGAGACAAAGATG-3′ F	24
	5′-CCGAATTGTCGATATCGCAT-3′ R	30

Table 2 Descriptive statistics of measured traits in male pupae obtained from back crossed colonies (H_1 generation)

Variable	Sample number	Mean	Standard error
Number of mother mite	117	0.89	0.32
Number of offspring mite	117	1.82	2.20
Total number of mite	117	2.71	2.31
Phenotypic score	104	0.5	0.5
Fecundity	104	5.05	2.23
Infestation	117	0.89	0.32

The UN391 locus with a physical position of 6.922 Kb is located on chromosome 7. This locus is located in the distance between GB11764 (6.752 Kb) and GB13873 (6.479 Kb) genes. In the study of Behrens *et al.* (2011), the GB11764 gene is located directly at the peak of the LOD score on chromosome 7, where the orthologous of the foxo gene is responsible for transcribing the insulin signaling pathway. This gene affects the body's response to growth, immune response, longevity, nutrition, cell death, and energy metabolism (Nijhout, 2003; Wu and Brown, 2006). For example, in Drosophila (Junger *et al.* 2003), Colex Muscovite (Sim and Denlinger, 2008) and in humans (Willcox *et al.* 2008). Therefore, researchers consider the foxo gene to be a suitable candidate gene for a trait expressed in the developmental stage of bees (Behrens *et al.* 2011).

The second, GB13873, is the orthologous of the Drosophila gene, futsch. Navajas *et al.* (2008) in a study of expression at the genomic level using microarrays found that this gene is significantly reduced (0.86 times) in the Varroa-resistant bee line compared to the susceptible line. Reports in Drosophila also indicate that this gene is downregulated in non-neural tissue at the growth stage and is involved in phosphorylation and stimulation of synaptic plasticity in neurons (Hummel *et al.* 2000).

Interestingly, most of the genes that were expressed differently in resistant and sensitive lines in the study by Navajas *et al.* (2008) were those involved in neuronal development and sensitivity.

Behrens *et al.* (2011) consider these two genes as promising candidates for the mite reproductive suppression trait, but at the same time emphasize that they cannot be fully attributed to this trait or a suitable biological explanation for it. In the present study, the position of UN391, which is located between the two above-mentioned candidate genes, has shown a significant relationship with a number of traits. Considering the data in Table 3, it can be concluded that the relationship between this position and the measured traits is significant and the results are consistent with the findings of Behrens *et al.* (2011).

The locus of HQ7622 was strongly associated with all measured traits. In this test, this locus has a significant relationship with at least two of the 5 measured traits, which indicates the importance of this position in the development of *Varroa* mite reproductive disorder. This locus is located on chromosome 7 of the bee and has a physical position of 4.135 Kb (Behrens *et al.* 2011). In the study of Behrens *et al.* (2011), the relationship between this locus and the phenotype of mite reproduction disorder in male bee pupa cells was not significant, but as mentioned in this study, among the loci studied; this locus has the most association with case traits that were viewed.

Successful reproduction of Varroa mites within host larval cells is critical to balancing the parasite-host relationship. The most obvious example is the primary mite host, *Apis cerana*, in which reproduction is limited to male larvae (Boot *et al.* 1999) and reproductive barriers exist between different host and parasite haplotypes (Navajas *et al.* 2008).

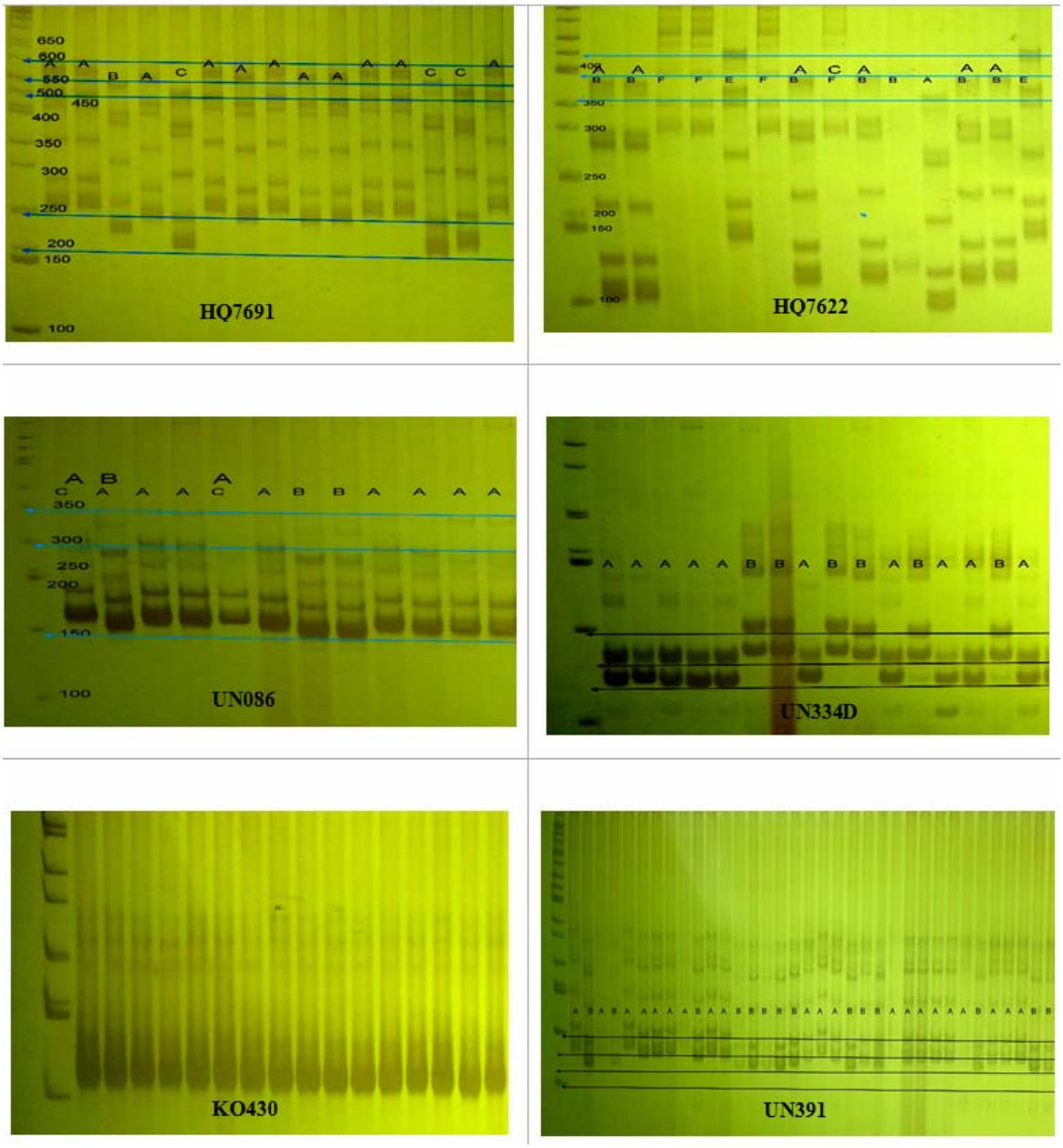

Figure 2 Variety of samples in different locations using polyacrylamide gel

Table 3 P- value of different traits in relation to different loci, calculated from a Kruscal-Wallis Test

	Number of mother mite	Number of offspring mite	Total number of mite	Phenotypic score	Fecundity	Infestation
HQ7622	0.0002	0.0127	0.0076	0.0236	0.0127	0.0002
HQ7691	0.0461	0.2746	0.1573	0.3483	0.2932	0.0461
UN086	0.4113	0.2892	0.2624	0.6309	0.3077	0.4113
UN334d	0.0370	0.1658	0.0579	0.8239	0.4110	0.0370
UN391	0.0200	0.0044	0.0009	0.1219	0.0414	0.0200

Preventing the reproductive success of female Varroa mites has been identified as an important factor in the resistance of African bees to this pest (Rosenkranz, 1999). It has also been shown to be present in a European population belonging to the island of Gotland (Locke and Fries, 2011). The results of this study showed that there is a good diversity in terms of some elements of the trait of reproductive disorder of Varroa mite, including fecundity and phenotypic score in a segregation population. Also there is a good relationship between some studied markers and the SMR traits. In a similar study, a guanine/adenine polymorphism at the honey bee SNP 9–9224292 has been described in honey bees (*A. mellifera*) in North America and New Zealand. The guanine allele at this SNP was associated with VSH behavior, which is a variable trait innate to honey bees that can help control the destructive mite *V. destructor* (Sainsbury *et al*. 2022). Also, Sepehri *et al*. (2023) concluded that the existence of Specified SNPs in three regions of the NorpA2 gene promoter be-tween the case-control groups that can be used in the molecular identification of Varroa-resistant colonies and breeding programs to produce Varroa-resistant colonies. It is therefore, possible that within the genomic structure of the bee made a choice for this trait and these markers shows promise for marker assisted selection of Iran honey bees when aiming for innate Varroa control traits.

Another advantage of using this trait in selective breeding programs is that by analyzing the reproductive success of mites inside bee larvae, the effect of these programs on the phenotype can be directly controlled. Therefore, the choice for this trait will be very useful in breeding programs to resist Varroa. Because several genes have major effects on this trait and individuals' genomes can be easily screened, marker-assisted selection (MAS) of this trait is much easier than other (often complex) behavioral traits that will be measurable on diploid worker bees.

It is strongly suggested that the benefits of haploid males be used in genotypic and phenotypic evaluations and gene mapping studies. It is also recommended to use males routinely in marker selection in breeding programs. Using haploid males, a more accurate answer can be reached faster.

CONCLUSION

On this basis, a summary of results obtained from present research shows that SMR is an important section of resistance of bee colonies to Varroa destructor mites. Interestingly, HQ7622 microsatellite marker was associated with all measured traits. In detail, the effect of the HQ7691 locus on the number of mother mites and the rate of mite infestation was significant. Furthermore, UN086 didn't affect any of the measured phenotypic characteristics. Using phenotypic and genomic DNA markers knowledge and creating of segregated population using of backcross mating between a susceptible × resistant colony, we demonstrated significant association between SMR trait and some of the investigated genetic markers. On this basis, we demonstrated the importance of certain microsatellite markers for genetically identification of bee colony resistance to *Varroa destructor* mites.

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